Elastin Fibers Display a Versatile Microbril Network in Articular Cartilage Depending on the Mechanical Microenvironments

Elastin Fibres Display a Versatile Microfibril Network in Kangaroo Articular Cartilage Depending on the Mechanical Microenvironments

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Running title: Elastin fibres in Kangaroo Articular Cartilage

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Elastin fibres are major extracellular matrix macromolecules that are critical in maintaining the elasticity and resilience of tissues such as blood vessels, lungs and skins. However, the role of elastin in articular cartilage is poorly defined. The present study investigated the organisation of elastin fibre in articular cartilage, its relationship to collagen fibres and the architecture of elastin fibres from different mechanical environments by using a kangaroo model. Five morphologies of elastin fibres were identified: straight fibre, straight fibre with branches, branching fibres directly associated with chondrocyte, wave fibre and fine elastin. The architecture of the elastin network varied significantly with cartilage depth. In the most superficial layer of tibial plateau articular cartilage, dense elastin fibres formed a distinctive cobweb-like meshwork which was parallel to the cartilage surface. In the superficial zone, elastin fibres were well organized in a preferred orientation which was parallel to collagen fibres. In the deep zone, no detectable elastin fibre was found. Moreover, differences in the organization of elastin fibres were also observed between articular cartilage from the tibial plateau, femoral condyle and distal humerus. This study unravels the detailed microarchitecture of elastin fibres which display a well-organized three-dimensional versatile network in articular cartilage. Our findings imply that elastin fibres may play a crucial role in maintaining the integrity, elasticity and the mechanical properties of articular cartilage, and that the local mechanical environment affects the architectural development of elastin fibres.

**Keywords:** articular cartilage; collagen fibres; elastin fibres, mechanical environment

Elastin fibres are macromolecules comprising an amorphous core of extensively cross-linked
elastin, surrounded by fibrillin-rich microfibrils.\textsuperscript{1-3} They are abundant in the extracellular matrix (ECM) of many tissues such as connective tissue proper, elastin cartilage and fetal tissue, as well as in organs such as skin, lungs, arteries, veins. The prevailing mechanical functions of elastin fibres are thought to endow the tissues with the critical properties of elasticity and resilience.\textsuperscript{4, 5} Increasing studies have shown that elastin fibres also play a critical role in regulating arterial development via controlling proliferation of vascular smooth muscle and stabilizing arterial structure;\textsuperscript{4} mediating disease states of supravalvular aortic stenosis (SVAS)\textsuperscript{6},\textsuperscript{7} and Williams syndrome;\textsuperscript{8} and enhancing the mechanical integrity of the lumbar annulus fibrosus.\textsuperscript{9}

The role of elastin in articular cartilage is poorly defined as early histochemistry studies suggested that little elastin was present in articular cartilage.\textsuperscript{5, 10-12} In articular cartilage, water, collagen matrix and proteoglycans (PGs) have been recognised to be the most influential elements in maintaining the mechanical functions of articular cartilage.\textsuperscript{13-16} A recent study using nonlinear optical microscopy has shown elastin-like fibres in bovine articular cartilage,\textsuperscript{17} and further immunohistochemistry studies confirm the identity of elastin fibres in bovine\textsuperscript{17, 18} and equine\textsuperscript{19} articular cartilage. Although the fibres are reported to occupy the superficial zone of articular cartilage, the arrangement and function of elastin fibres in the articular cartilage, especially of different joints with distinct mechanical environments, remain to be elucidated.

By using multiphoton microscopy and sulforhodamine B (SRB) which stains elastin fibres,\textsuperscript{20} this study aims to examine the elastin fibre network within kangaroo articular cartilage and their relationship with the mechanical function of articular cartilage. We directly confirmed that the two-photon fluorescence (TPF) signal was of elastin fibre and they had a close
relationship with the collagen matrix and chondrocytes. We also found that the architecture and content of the elastin fibre network varied significantly with cartilage depth and with the mechanical environments of articular cartilage.

METHODS

Sampling
Six forelimb and six hindlimb joints were obtained from six male kangaroos aged at approximately 5 years. Articular cartilage from the tibial plateau, femoral condyle and distal humerus was inspected for the absence of macroscopic degeneration. Cylindrical cartilage samples (3 plugs from each site) were harvested using 5 mm diameter punches from the medial tibial plateau (meniscus covered area), the weight-bearing areas of the medial femoral condyle and medial distal humerus (squares in Fig. 1). The cartilage plugs were sliced into samples corresponding to the superficial and deep zone.

Articular Cartilage Staining
For imaging elastin, articular cartilage sample was stained with SRB as previously described. Briefly, the samples were stained in 1 mg/ml SRB solution for 1 minute. After a thorough wash in PBS (PBS, pH 7.2), the cartilage samples were mounted between a coverslip and a glass slide.

Imaging
Images of the elastin and collagen were acquired by a multiphoton confocal laser scanning microscope (Leica TCS SP2 acousto-optical beam splitter) (AOBS) equipped with several light sources, including a diode-pumped solid-state (DPSS) laser at 561 nm and the multiphoton laser used in this study. The multiphoton laser was generated by a Spectra Physics Mai Tai
sapphire system and could be tuned from 710 to 990 nm. As a comparison to previous reported
elastin-like fibres from TPF signal, an unstained cartilage sample was excited by a laser of
890 nm. The TPF signal was collected in the epi-direction at 500 to 550 nm emission
wavelength and the second harmonic generation (SHG) signal from collagen was collected in
the forward direction at 445 nm. For SRB stained samples, the DPSS laser at 561 nm was used
and the multiphoton laser at 890 nm. The SHG signal from collagen was acquired at 445 nm
and the SRB fluorescent signal from elastin was acquired at 565-590 nm emission wavelength.
All images were acquired as a series of stacks at an imaging step size of 0.5 μm.

Fibre Orientation Analysis

Digital image analysis software ImageJ (NIH, Maryland, USA) was used to conduct the image
analysis. The orientation of the elastin fibres and the collagen fibres was analysed using
OrientationJ (an ImageJ-plug-in) which was validated to study the collagen orientation in a
previous study. The hue-saturation-brightness (HSB) colour coded images, which used
colours to show the orientation of an object, were generated from the images acquired by
microscopy. The relative orientation of the elastin fibres and collagen fibres was plotted and
their predominant orientation and coherency value were calculated. The image stacks of the
elastin and collagen fibres acquired at the same region were merged and reconstructed into
three-dimensional (3D) images to study their spatial relationship.

RESULTS

Verification of the Elastin Fibre in Articular Cartilage

To confirm the presence of elastin fibre in articular cartilage, unstained articular cartilage of
kangaroo tibial plateau was firstly imaged using a multiphoton confocal laser scanning
microscope and was later stained for SRB excitation. From an unstained surface of articular cartilage, two signals were collected: SHG indicating the collagen fibre network and TPF representing an additional fluorescent fibre network which did not overlap with the collagen fibres (Fig. 2A and B). For the SRB stained articular cartilage sample, three signals, TPF, SHG and SRB were collected (Fig. 2C-E). The elastin fibre network shown in the SRB fluorescent image (Fig. 2E) was similar to that in the TPF image (Fig. 2C) and the montage of the left half part of TPF image and the right half part of SRB image showed a perfect match between the TPF image and SRB image in terms of fibre extension (Fig. 2F). A merged image of TPF and SHG (Fig. 2G) and a merged image of SRB and SHG (Fig. 2H) further confirmed the identity of elastin fibres. The comparison of TPF and SRB images indicated a higher resolution and less digital noise in SRB stained fluorescent images.

**Morphological Characteristics of Elastin Fibres in Articular Cartilage**

As visualized by SRB fluorescence, five morphologically different elastin fibres were found in tibial plateau articular cartilage of kangaroo: straight fibre with branches in the ECM (Fig. 3A and C), branching fibres associated with chondrocyte (squared in Fig. 3D, Fig. 3E), straight fibre in the ECM (Fig. 3F), wave fibre in the ECM (Fig. 3B) and fine elastin on the cell surface and in the pericellular matrix (Fig. 3G-J). Elastin fibres with branches were most abundant and formed a complex three dimensional network by cross-linking extensively in the same plane and branching into different layers (Fig. 3A and C). Straight elastin fibres represented the second abundant type and they were highly oriented and parallel to each other (Fig. 3F). Fine elastin was found in the pericellular matrix (arrow in Fig. 3G-J) and the surface of chondrocytes (arrow head in Fig. 3H) where no individual fibre was resolvable (Fig. 3G-J). A few wave fibres
representing a relax status were also found in the ECM of kangaroo articular cartilage (Fig. 3B).

Branching fibres directly associated with chondrocyte represented the least common type of elastin fibres in the articular cartilage of kangaroo tibial plateau (square in Fig. 3D, Fig. 3E). As shown, a relative bigger elastin fibre stem (arrowhead in Fig. 3E) was directly associated with a chondrocyte (square in Fig. 3E), and smaller branches (arrow in Fig. 3E) extended from the stem to various directions which formed a complex three-dimensional root-like structure.

**Variation of Elastic Network with Depth**

In the most superficial layer of tibial plateau articular cartilage, from the articular surface down to around 3μm depth, the elastin fibres ran parallel to the articular surface and were organized in a cobweb-like structure with extensive cross-linking to one another (Fig. 4A-C). Beneath the most superficial layer, the elastin fibres in the superficial zone showed a preferred orientation and ran parallel to the long axis of the chondrocytes (Fig. 4D-F). In the deep zone, no individual elastin fibres could be resolved but dense and fine elastin was found in the pericellular matrix and on chondrocyte surfaces (arrow in Fig. 4G-I).

**The Structural Relationship between the Elastin and Collagen Fibres**

The confocal fluorescent images (Fig. 5A and B) and the corresponding HSB-colour-coded images (Fig. 5 D and E) indicated that the elastin fibres were randomly distributed but the collagen fibres were highly oriented in the most superficial layer of tibial plateau articular cartilage. Quantitative study showed that the elastin fibres were distributed more widely in all directions (Fig. 5C) with a lower coherency of 17% and a predominant degree of -71° (Fig. 5H).

In contrast, the orientation of the collagen fibres in the same region was more isotropic with higher coherency of 51% and a dominant degree of 25° (Fig. 5F and H). The merged image (Fig.
5G) and 3 dimensional reconstruction image (Fig. 7A) further confirmed the coarse distribution of elastin fibres but highly oriented collagen fibres in the most superficial layer.

In the superficial zone, the elastin fibres (Fig. 6A and B) mostly ran parallel to the collagen fibres (Fig. 6D and E). Quantitative study showed that the elastin fibres and the collagen matrix had similar distribution ranges, close predominant degrees and coherency values (Fig. 6C, F and H). The merged image (Fig. 6G) and three dimensional reconstruction image (Fig. 7B) also indicated that both the elastin fibres and the collagen fibres were highly oriented in a similar direction.

**Variation of Elastin Fibres with Joints**

The variation between joints in terms of morphology and structure of the elastin fibre networks was most significant in the most superficial layer. In the most superficial layer of articular cartilage, the elastin fibres were large, coarse and heavily cross-linked without a preferred orientation in tibial plateau (Fig. 8A); were also coarse but showed a predominant orientation in femoral condyle (Fig. 8B); and were finer and shorter in distal humerus (Fig. 8C).

Compared to the most superficial layer, less elastin fibres with more clear orientation were found in the superficial zone of all the joints studied (Fig. 8D-F). However, the elastin fibre variation between joints was not obvious except the seemingly larger fibres found in the tibial plateau articular cartilage (Fig. 8D).

**DISCUSSION**

The present study shows that elastin exists in the ECM of kangaroo articular cartilage. The elastin fibres in the superficial zone are integrated as a three-dimensional network which varies
significantly with depth of articular cartilage and different joint of kangaroo. Given the significant volume and complexity of the elastin network, it is suggested that elastin fibres could have both biochemical and biomechanical roles.

The elastin fibre system has a close relationship with other components of tissues. Early investigations have found an association between elastin fibres and PGs in dermis, cultured smooth muscle cell and skin. Bridging molecules were also identified to anchor elastin fibres to collagens (e.g. MP78/70, the α1 chain of type VIII collagen) and cell surface (e.g. Fibulin-5). We revealed that the elastin fibres in articular cartilage were closely related to collagen (Fig 5, 6, and 7) and that some elastin fibres were anchored to chondrocytes (Fig. 2D and E). Additionally, the cross-linking of massive elastin fibres themselves in the same plane and branching into different depths (as shown in Fig. 3A-E and Fig. 4A-F) contribute a three dimensional elastic network for the articular cartilage surface. Thus, along with other ECM components, elastin fibres could also play a crucial role in structural support to the articular cartilage.

The varied architecture of elastin with cartilage depth could have significant biomechanical implications. Firstly, fine and dense elastin found around the chondrocytes (Fig. 2D-E, G-J) forms a niche and may provide a crucial microenvironment where chondrocytes suffer only a mild stretching despite the vigorous forces applied to the articular cartilage. The cobweb-like elastin fibre network found in the most superficial layer could render the articular cartilage surface more resistant to stretches in different directions, as previous studies have shown that tensile strain predominates near the surface with hydrostatic compressive forces dominating below the surface layers. Moreover, the elastin fibres in the superficial zone were found to
be parallel to the adjacent collagen matrix and interdispersed in the collagen matrix (Fig. 6, Fig. 7B). Whether the elasticity and resilience properties of elastin fibres could enable a protective mechanism to collagen under harsh shear stresses is unclear from this study, but this finding merits further investigation to reveal the detailed relationship between elastin fibres and collagen matrix in articular cartilage under stress.

Previous studies in other tissues have showed that mechanical stimuli affect the development of elastin fibres. Cyclical mechanical stretches upregulate gene expression of elastin in human lamina cribrosa cells in vitro; mechanical stress could induce the deposition of fibrillin-1 in vitro; and pressure simulating orthodontic force could upregulate the tropoelastin gene. The current study revealed variations in elastin fibre network between tibial plateau, femoral condyle and distal humerus articular cartilage (Fig. 8). This variation with different types of joints implies a potential relationship with the local mechanical environments. Further studies, however, are essential in order to learn whether mechanical stimuli also affect the development of the elastin fibre network in articular cartilage.

Marfan syndrome in humans is caused by mutations in the FBN1 gene which encodes fibrillin-1 protein that is essential for the proper formation and biogenesis of elastin fibres. Marfan syndrome develops signs of abnormal joint flexibility, and is linked to early osteoarthritis. Our findings of elastin fibres in articular cartilage might provide novel insights into the importance of elastin fibers in skeletal anomalies, such as osteoarthritis.

In conclusion, the present work assessed the microstructural characteristics of elastin fibres in kangaroo articular cartilage. A well organized three dimensional elastin network was revealed in the ECM of articular cartilage surface as well as surrounding the chondrocyte
surface and in the pericellular matrix. The architecture of elastin fibres may provide novel insights into the role of the elastin network in articular cartilage and cartilage degeneration. Changes of elastin fibres in diseased articular cartilage, such as osteoarthritic cartilage, will be an important subject of further investigation.

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References


**Figure legends:**

**Fig. 1.** A photograph of the tibial plateau, femoral condyle and distal humerus of a kangaroo. Images were obtained from the meniscus covered articular cartilage of medial tibial plateau (square area in A), the medial femoral condyle (square area in B) and the medial distal humerus (square area in C).

**Fig. 2.** Verification of elastin fibres in the tibial plateau articular cartilage of kangaroo. Distinctive fibre networks in TPF image (A) and collagen matrix in SHG image (B) were found on the cartilage surface of an unstained sample. In the SRB stained articular cartilage sample, fibres found in the TPF image (C) were identical to elastin fibres in SRB image (E); collagen matrix is imaged by its SHG signal (D). A montage image (F) and merged images (G and H) confirmed the fibres in TPF were elastin fibres (Green = TPF and Red = SHG in G; Green = SRB and Red = SHG in H).

**Fig. 3.** Representative morphology of elastin fibres stained with SRB in the tibial plateau
articular cartilage of kangaroo: straight elastin fibres with branches (A and C); branching elastin fibres associated with chondrocyte (square in D; and E,); straight elastin fibres (F); wave elastin fibres (B); and fine elastin (G–J, arrow indicated fine elastin in the pericellular matrix, arrow head indicated fine elastin on the chondrocyte surface).

**Fig. 4.** Variation of elastin fibre with depth in the tibial plateau articular cartilage of kangaroo.

In the most superficial layer, elastin fibres were extensively cross-linked without a predominant orientation (A-C). Underneath the most superficial layer, elastin fibres were generally oriented in one direction in the superficial zone (D-F). In the deep zone, fine elastin was presented on the chondrocyte surface (indicated as arrow head in H) and in the pericellular matrix (indicated as arrow in G–I).

**Fig. 5.** Orientation analysis showing the organization of elastin fibres was different from that of collagen matrix in the most superficial layer of tibial plateau articular cartilage.

**Fig. 6.** Orientation analysis showing that elastin fibres were generally parallel to collagen matrix in the superficial zone of tibial plateau articular cartilage.

**Fig. 7.** Three-dimensional reconstruction of cartilage surface showing the relationship between elastin fibres and collagen fibres in the most superficial layer (A) and superficial zone (B). Green = elastin fibres; Red = collagen.

**Fig. 8.** Organization of elastin fibres in articular cartilage differed between from the tibial plateau (A and B), femoral condyle (C and D) and distal humerus (E and F). (stain: SRB)
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Fig 7.

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*Scale bar: 50 μm*